A Triangular Platinum(II) Multi-nuclear Complex with Cytotoxicity Towards Breast Cancer Stem Cells

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Dedication ((optional))

Abstract: The preparation of multi-nuclear metal complexes offers a route to novel anticancer agents and delivery systems. The potency of a novel triangular multi-nuclear complex containing three platinum atoms, **Pt-3**, towards breast cancer stem cells (CSCs) is reported. The tri-nuclear platinum(II) complex, **Pt-3** exhibits selectivity toxicity towards breast CSCs over bulk breast cancer cells and non-tumorigenic breast cells. Remarkably, **Pt-3** inhibits the formation, size, and viability of mammospheres to a better extent than salinomycin, an established CSC-potent agent, and cisplatin and carboplatin, clinically used platinum drugs. Mechanism of action studies show that **Pt-3** effectively enters breast CSCs, penetrates the nucleus, induces genomic DNA damage, and prompts caspase-dependent apoptosis. To the best of our knowledge, **Pt-3** is the first multi-nuclear platinum complex to selectivity kill breast CSCs over other breast cell types.

Platinum(II)-based anticancer drugs, cisplatin, carboplatin, and oxaliplatin, are used worldwide, singularly or in conjunction with other chemotherapeutic agents, to treat various types of cancers.[1] The therapeutic effect of the platinum(II) agents is largely attributed to their ability to covalently bind DNA and distort its structure.[2] This prevents DNA replication and transcription, and trigger programmed cell death.[3] Despite their success, these platinum(II) drugs have significant drawbacks including systemic toxicity and side-effects due to their inability to distinguish between proliferating cancer cells and fast-growing non-tumorigenic cells, acquired or inherent resistance leading to ineffective treatment against several tissue types, and the failure to prevent cancer reoccurrence.[4] The latter is believed to be related to the existence of cancer stem cells (CSCs), a sub-population of tumour cells with the ability to differentiate, self-renew and seed the formation of new tumours.[5] The platinum(II) drugs are unable to effectively remove CSCs (of any tissue type) at their clinically administered doses.[6] This is primarily due to elevated levels of DNA repair-linked effectors (such as BRCA1, ATR, ATM, and Chk1) and platinum-related drug efflux pumps (such as ATP-binding cassette transporters) in CSCs.[7] Indeed, several independent in vitro and in vivo studies have shown that cisplatin, carboplatin, and oxaliplatin, all enrich rather than deplete, CSCs in heterogeneous tumour populations.[8] Therefore there is a clear need for the development of novel platinum agents that can remove both bulk cancer cells and CSCs at clinically relevant concentrations.

Multi-nuclear inorganic structures with well-defined geometry and size can be readily prepared.[9] The application of such macromolecular entities in cancer research has increased over the last two decades.[10] A triple helicate with two iron(II) ions and three bisazopyridine ligands was shown bind to three-way duplex DNA junctions and exhibit reasonable activity against a range of bulk cancer cell lines.[11] Independently, the same helicate with two nickel(II) ions, instead of two iron(II) ions, was reported to bind tightly to G-quadruplex DNA and preferentially reduce breast CSC growth over bulk breast cancer cells.[12] Structurally similar di-ruthenium(II) double-stranded complexes displayed up to 100-fold greater potency (sub-micromolar range) for bulk breast cancer cells than cisplatin whereas an analogous di-ruthenium(II) triple-stranded helicate exhibited significantly lower activity (micromolar range).[13] More recently, di-iron(II) helicate-like architectures, prepared via diastereoselective self-assembly, were shown to kill bulk colon cancer cells lacking p53 (a vital tumour suppressor) in the nanomolar range, with nearly 1000-fold greater potency than non-cancerous retinal pigment epithelial cells.[14] Remarkably, cytotoxicity studies with di-ruthenium(II) double-stranded helicate and mesocate complexes showed that the former favorably killed bulk colon cancer cells lacking p53 and the latter killed bulk colon cancer cells possessing p53.[15]

Multi-nuclear platinum(II) complexes have been widely studied as potential anticancer agents and drug delivery vehicles.[1c, 16] Notably, the tri-nuclear complex, [trans-diamminechloroplatinum(II)][μ-trans-diamminebis(hexanediamine)platinum(II)] nitrate (BBR3464) was shown to covalently bind DNA, induce atypical DNA lesions that evade DNA repair, and prompt cell death in cisplatin-resistant cell lines.[17] Despite clinical trials suggesting partial response in non-small cell lung cancer and advanced ovarian cancer patients, follow-up studies have not been reported.[18] The non-coordinating analogues of BBR3464, TriplatinNC and TriplatinNC-A exhibit strong DNA binding affinity and bulk cancer

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cell potency.[19] A hexanuclear platinum(II) assembly containing six platinum(II) centres and four 2,4,6-tris(4-pyridyl)-1,3,5-triazine ligands exhibited promising in vitro potency against a panel of bulk cancer cell lines.[20] The same platinum(II) cage was also successfully deployed as a delivery vehicle for platinum(IV) prodrugs into certain bulk cancer cells.[21] Despite the growing interest in studying the anticancer properties of multi-nuclear inorganic structures, none of the platinum(II) multi-nuclear complexes reported to date have been challenged with CSCs. Here we report a triangle-shaped, tri-nuclear platinum(II) complex with breast CSC potency and selectivity (over bulk breast cancer cells and non-tumorigenic breast cells). This is, as far as we are aware, the first study to investigate the anti-CSC properties of a platinum(II) multi-nuclear complex.

The tri-platinum(II) complex, **Pt-3** was prepared as outlined in Scheme S1. Specifically, **Pt-3** was prepared by reacting equimolar amounts of Pt(1,1-bis(diphenylphosphino)ethylene)(OSO_2CF_3)$_2$ (**Pt-1a**), benzotriazole, and sodium methoxide in DMF at 80 °C for 3 h. Methanol was added to the resultant solution to remove impurities as precipitates, and diethyl ether was triturated into the filtrate to yield pure **Pt-3** as a white solid. Purified **Pt-3** was fully characterised by ^{1}H NMR and infrared spectroscopy, high-resolution ESI mass spectrometry, and elemental analysis (see ESI, Figure S1-4). Single crystals (colourless blocks) of **Pt-3** suitable for X-ray diffraction studies were obtained by slow diffusion of diethyl ether into a methanol:DMF (9:1) solution of **Pt-3** (CCDC 1906805, Figure 1 and Table S1). Selected bond distances and bond angles data are presented in Table S2. The structure consists of three platinum(II) centres, each coordinated to two phosphorous atoms belonging to 1,1-bis(diphenylphosphino)ethylene and two nitrogen atoms from two separate benzotriazole ligands. The complex, **Pt-3** is tricationic and crystallizes with three triflate counter anions and one molecule of DMF. The average P–Pt–P bite angle is 85.22° and the average N–Pt–N bite angle is 85.43°. This shows that each platinum(II) centre adopts a pseudo square-planar geometry. The average Pt–N (2.08 Å) and Pt–P (2.24 Å) bond distances are consistent with bond parameter for related platinum(II) complexes.[22] The adjacent Pt-Pt distances vary from 5.882 to 5.918 Å, and the Pt(2)–Pt(1)–Pt(3) angle = 59.71°, Pt(2)–Pt(3)–Pt(1) angle = 59.98°, and Pt(1)-Pt(2)-Pt(3) angle = 60.31. Therefore the three platinum atoms are arranged in an equilateral triangular orientation relative to each other.

By reacting benzotriazole and sodium methoxide with two equivalence of Pt(1,1-bis(diphenylphosphino)ethylene)(OSO_2CF_3)Cl (**Pt-1b**) in DMF at 80 °C for 3 h, the di-nuclear platinum(II) complex, **Pt-2** was isolated (Scheme S2). The di-nuclear platinum(II) complex, **Pt-2** was fully characterised by standard spectroscopic and analytical methods (see ESI, Figure S3, S5). Single crystals (colourless) of **Pt-2** suitable for X-ray diffraction studies were obtained by slow diffusion of diethyl ether into a DCM:DMF (9:1) solution of **Pt-2** (CCDC 1906803, Figure 2 and Table S1). Selected bond distances and bond angles data are presented in Table S3. The structure consists of two platinum(II) centres, each coordinated to two phosphorous atoms belonging to 1,1-bis(diphenylphosphino)ethylene, one nitrogen atom from benzotriazole, and one chloride ligand. Akin to **Pt-3**, the platinum(II) centres in **Pt-2** adopt a pseudo square-planar geometry, with the average P–Pt–P bite angle being 85.65° and the average N–Pt–Cl bite angle being 89.95°. The dihedral angle between the P(2)P(1)Pt(1)Cl(1)N(1) and P(3)P(4)Pt(2)Cl(2)N(3) is 130.38°. The average Pt–N (2.10 Å), Pt–P (2.24 Å), and Pt–Cl (2.35 Å) bond distances are consistent with bond parameter for related platinum(II) complexes.[22]

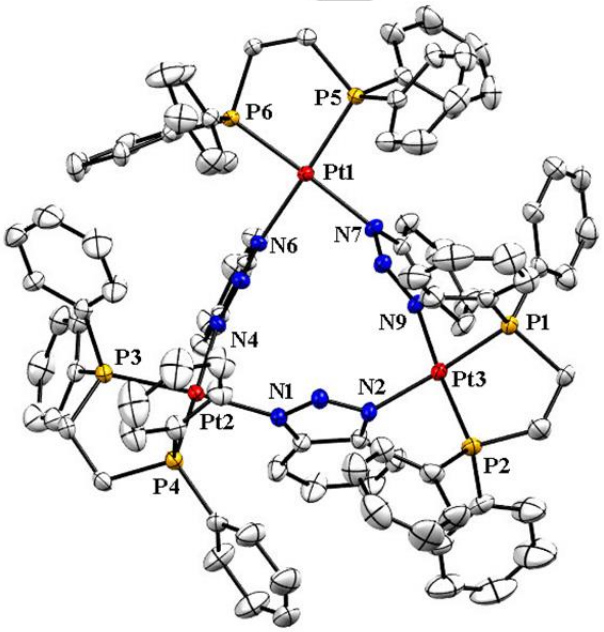

Figure 1. X-ray structure of the triangular, tri-nuclear platinum(II) complex, **Pt-3** comprising of three Pt(1,1-bis(diphenylphosphino)ethylene) moieties and three deprotonated benzotriazole ligands. Ellipsoids are shown at 30% probability, C in grey, N in dark blue, P in yellow, and Pt in red. H atoms, co-crystallizing triflate counter anions, and solvent molecules have been omitted for clarity.

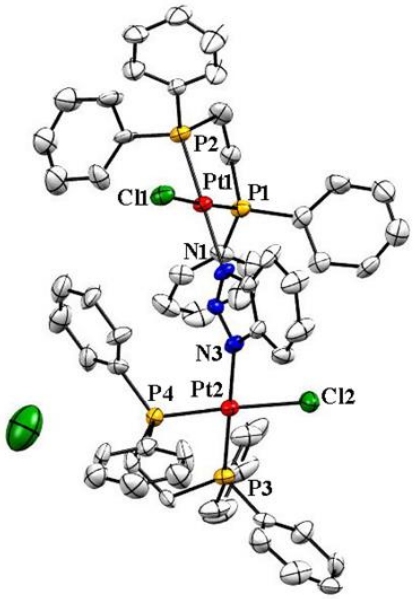

Figure 2. X-ray structure of the di-nuclear platinum(II) complex, **Pt-2** comprising of two Pt(1,1-bis(diphenylphosphino)ethylene) moieties and one deprotonated benzotriazole ligand. Ellipsoids are shown at 30% probability, C in grey, N in dark blue, P in yellow, Cl in green, and Pt in red. H atoms have been omitted for clarity.

The cytotoxicity of the tri-nuclear platinum(II) complex, **Pt-3**, along with its mono-nuclear (**Pt-1a**) and di-nuclear (**Pt-2**) analogues, towards breast CSC-enriched HMLER-shEcad cells and CSC-depleted HMLER cells was determined using the MTT assay. The IC_{50} values were determined from dose-response curves (Figure S6-8) and are summarised in Table 1. The tri-nuclear platinum(II) complex, **Pt-3** exhibited micromolar potency towards both cell lines, comparable to salinomycin (an established breast CSC-potent agent).[23] Notably, **Pt-3** exhibited 2-fold greater potency (p < 0.01, n = 18) for HMLER-shEcad cells over HMLER cells (Figure S8). The mono- and di-nuclear complexes, **Pt-1a** and **Pt-2**, also exhibit micromolar potency towards bulk breast cancer cells and breast CSCs, but do not display CSC selectivity (Figure S6-7). This suggests that the multi-nuclear structure of **Pt-3** contributes to CSC-selective potency. As expected the anticancer platinum(II) drugs, cisplatin and carboplatin, exhibited preferential potency for bulk breast cancer cells over breast CSCs (Table 1 and Figure S9-10). As a measure of therapeutic potential, the cytotoxicity of **Pt-3** towards normal human epithelial breast MCF710A cells was determined. The complex, **Pt-3** was 2-fold less potent toward MCF710A cells (IC_{50} value = 2.59 ± 0.12 μM, Figure S11) than HMLER-shEcad cells, indicating selective toxicity for breast CSCs over non-tumorigenic breast cells.

Table 1. IC_{50} values of the platinum(II) complexes, **Pt-1a**, **Pt-2**, **Pt-3**, cisplatin, carboplatin, and salinomycin against HMLER cells, HMLER-shEcad cells, and HMLER-shEcad mammospheres.

Compound	HMLER IC_{50} [μM][a]	HMLER-shEcad IC_{50} [μM][a]	Mammosphere IC_{50} [μM][b]
Pt-1a	5.01 ± 0.03	7.01 ± 0.06	14.50 ± 0.91
Pt-2	2.59 ± 0.09	2.35 ± 0.01	16.00 ± 0.56
Pt-3	2.24 ± 0.01	1.26 ± 0.03	4.55 ± 0.02
cisplatin	2.57 ± 0.02	5.65 ± 0.30	13.50 ± 2.34
carboplatin	66.11 ± 0.50	72.59 ± 0.09	18.06 ± 0.40
salinomycin [c]	11.43 ± 0.42	4.23 ± 0.35	18.50 ± 1.50

[a] Determined after 72 h incubation (mean of three independent experiments ± SD). [b] Determined after 5 days incubation (mean of three independent experiments ± SD). [c] Reported in references 23a and 24.

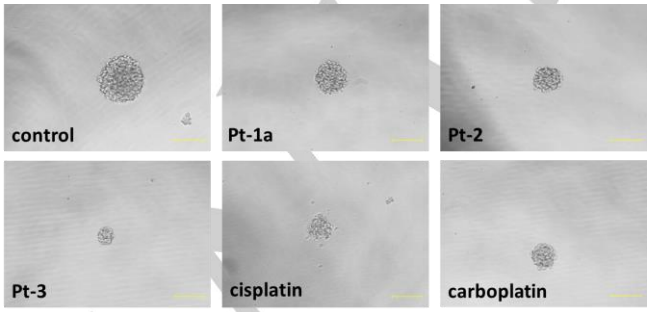

Figure 3. Representative bright-field images (× 20) of HMLER-shEcad spheroids in the absence and presence of **Pt-1a**, **Pt-2**, **Pt-3**, cisplatin, and carboplatin at their respective IC_{20} values (5 days incubation).

Breast CSCs grown in serum-free media, under low-attachment conditions form three-dimensional, tumour-like structures called mammospheres. To reliably gauge the in vivo CSC potential of the platinum(II) complexes, **Pt-1a**, **Pt-2,** and **Pt-3**, the mammosphere formation assay was performed. Treatment of single cell suspensions of HMLER-shEcad cells with the platinum(II) complexes, **Pt-1a**, **Pt-2,** and **Pt-3** (IC_{20} value for 5 days) noticeably reduced the number and size of mammospheres formed (Figure 3 and S12). The greatest inhibitory effect was observed for **Pt-3** and it was comparable or better than the effect observed for salinomycin treatment (IC_{20} value for 5 days) (Figure 3 and S12-13). Addition of cisplatin and carboplatin (at their IC_{20} value for 5 days) also reduced the number and size of mammospheres formed, albeit to a lesser level than **Pt-3** (Figure 3 and S12). To determine the effect of **Pt-1a**, **Pt-2,** and **Pt-3** on mammosphere viability, the colorimetric resazurin-based reagent, TOX8 was used. All of the platinum(II) complexes displayed micromolar potency (Table 1 and Figure 4). Notably, the tri-nuclear platinum(II) complex **Pt-3**, displayed ≥3-fold greater potency for mammospheres than the mono- and di-nuclear complexes, **Pt-1a**, **Pt-2**, and cisplatin, and >4-fold greater potency than salinomycin[24] and carboplatin (Table 1 and Figure 4).

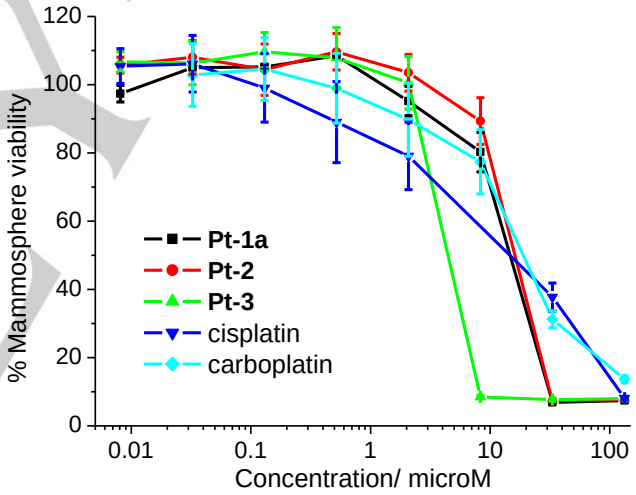

Figure 4. Representative dose-response curves for the treatment of HMLER-shEcad spheroids with **Pt-1a**, **Pt-2**, **Pt-3**, cisplatin, and carboplatin. The concentration of treated **Pt-1a**, **Pt-2**, **Pt-3**, cisplatin, and carboplatin was based on Pt concentration.

To shed light on the mechanism of CSC toxicity of the platinum(II) complexes further cell-based studies were conducted. Cellular uptake studies were carried out to determine breast CSC permeability. HMLER-shEcad cells were treated with **Pt-2**, **Pt-3**, and cisplatin at a non-lethal dose (2 μM for 24 h) and the internalized platinum content was determined by inductively coupled plasma mass spectrometry (ICP-MS). As depicted in Figure 5, the tri- and di-nuclear complexes, **Pt-3** and **Pt-2** (727.88 ± 13.25 and 579.47 ± 10.37 ppb of Pt/ million cells respectively) were taken up more readily than cisplatin (41.01 ± 0.62 ppb of Pt/ million cells). A clear correlation between HMLER-shEcad cellular uptake and lipophilicity (LogP values) of **Pt-2**, **Pt-3**, and cisplatin was observed (Table S4). The amount of **Pt-2**, **Pt-3**, and cisplatin entering breast CSC nuclei, and thus gaining access to genomic DNA, was also determined (Figure 5).

A reasonably large amount of internalized **Pt-3** was detected in the nucleus (15 %). Relatively lower levels of internalized **Pt-2** (8 %) and cisplatin (7 %) were recorded in breast CSC nuclei, suggesting that **Pt-3** has the greatest potential to damage genomic DNA. Taken together, the cellular uptake data suggests that **Pt-3**-mediated CSC death could be related to a genomic DNA-dependent pathway.

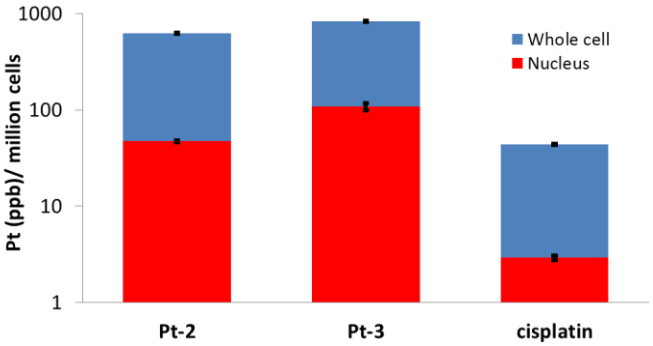

Figure 5. Platinum content in whole cell and nucleus fractions isolated from HMLER-shEcad cells treated with **Pt-2**, **Pt-3**, or cisplatin (2 μM for 24 h). The y-axis is given the logarithmic scale. Error bars = SD. The concentration of treated **Pt-2**, **Pt-3**, and cisplatin was based on Pt concentration.

As the tri-nuclear platinum(II) complex, **Pt-3** was shown to enter breast CSC nuclei, its potential to damage genomic DNA was probed by monitoring the expression of biomarkers related to the DNA damage pathway using immunoblotting methods. HMLER-shEcad cells incubated with **Pt-3** (0.5−1 μM for 72 h) displayed a marked increase in the expression of the phosphorylated forms of H2AX and CHK2, indicative of DNA damage (Figure S14).[25] Traditional anticancer platinum(II) complexes such as cisplatin bind covalently to genomic DNA.[3b] As the platinum(II) centres in **Pt-3** are bound to strongly coordinating ligands and given the reasonable stability of **Pt-3** in biologically relevant conditions (PBS, PBS with cellular reductant, and cell media) (Figure S15-17), the multi-nuclear complex is likely to, mainly, interact with genomic DNA in a non-covalent manner. ^{1}H NMR spectroscopy and ESI mass spectrometry studies were also performed to confirm the stability of the tri-nuclear platinum(II) complex, **Pt-3** in partially aqueous solution (see ESI, Figure S18-19).

To compare the ability of **Pt-3**, **Pt-2**, and cisplatin to covalently bind DNA, the ct-DNA precipitation assay was carried out (see ESI). The amount of platinum on ct-DNA increased in the following order **Pt-3** (16.63 ± 2.52 mg/ L) < **Pt-2** (138.13 ± 3.77 mg/ L) < cisplatin (212.16 ± 2.49 mg/ L). This is consistent with the presence of labile Pt-Cl bonds in cisplatin and **Pt-2** and not in **Pt-3**. To determine the non-covalent binding affinity and mode of **Pt-3** to DNA, ethidium bromide (a strong intercalator) and DAPI (a strong minor groove binder) displacement studies were carried out. Upon incremental addition of **Pt-3** (0 - 35 μM) to a solution of ct-DNA (20 μM) and ethidium bromide (1 μM), the emission associated to the ethidium bromide-DNA complex (originating from the intercalation of ethidium bromide between DNA base pairs) markedly decreased (Figure S20). Upon incremental addition of **Pt-3** (0 - 35 μM) to a solution of ct-DNA (20 μM) and DAPI (1 μM), the emission associated to the DAPI-DNA complex (originating from the binding of DAPI to the minor groove) decreased to a similar extent (Figure S21). Indeed the ethidium bromide quenching constant (K_q = 7.4 ± 0.1 × 10^4 M^{-1}) was similar to the DAPI quenching constant (K_q = 7.6 ± 0.2 × 10^4 M^{-1}). Control studies with **Pt-1a** and **Pt-2** (both 0 - 35 μM), revealed that **Pt-2** displaced ethidium bromide and DAPI from ct-DNA to a greater level than **Pt-1a** (Figure S20-21 and Table S5). This suggests that the intercalative and groove binding ability of **Pt-3** is facilitated by the Pt(1,1-bis(diphenylphosphino)ethylene) unit bound to benzotriazole rather than the Pt(1,1-bis(diphenylphosphino)ethylene) unit alone. Collectively this suggests that **Pt-3** binds to DNA non-covalently, and this probably triggers a DNA damage response.

Unrepaired DNA lesions can lead to apoptosis.[26] HMLER-shEcad cells exposed to **Pt-3** (0.5−2 μM for 72 h) displayed markedly higher levels of cleaved caspase 3 and 7 compared to untreated cells (Figure 6A), characteristic of caspase-dependent apoptosis. Cytotoxicity studies in the presence of z-VAD-FMK (5 μM), a peptide-based caspase-dependent apoptosis inhibitor showed that the potency of **Pt-3** towards HMLER-shEcad cells decreased significantly (p < 0.05, IC_{50} value = 2.20 ± 0.05 μM) (Figure 6B). This confirms that **Pt-3** induces caspase-dependent CSC death. As expected the potency of cisplatin, a well-known apoptosis-inducer, towards HMLER-shEcad cells decreased significantly (p < 0.05, IC_{50} value = 10.21 ± 0.78 μM) in the presence of z-VAD-FMK (5 μM) (Figure S22). Interestingly the potency of **Pt-1** and **Pt-2** towards HMLER-shEcad cells was not significantly altered (IC_{50} value of **Pt-1** = 9.14 ± 1.09 μM and IC_{50} value of **Pt-2** = 2.65 ± 0.31 μM) in the presence of z-VAD-FMK (5 μM), suggesting that these platinum(II) complexes act via a non-apoptotic mechanism (Figure S23-24). Overall the cellular studies show that **Pt-3** can enter the nucleus and induces genomic DNA damage, which ultimately leads apoptotic CSC death.

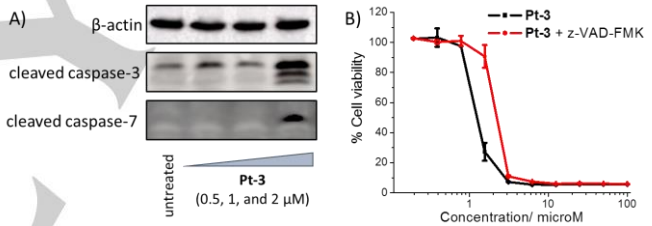

Figure 6. (A) Immunoblotting analysis of proteins related to the caspase-dependent apoptosis pathway. Protein expression in HMLER-shEcad cells following treatment with **Pt-3** (0.5, 1, and 2 μM for 72 h). (B) Representative dose-response curves of **Pt-3** against HMLER-shEcad cells in the absence and presence of z-VAD-FMK (5 μM) after 72 h incubation. The concentration of treated **Pt-3** was based on Pt concentration.

In summary we show that a triangle-shaped platinum(II) metallacycle, **Pt-3**, exhibits impressive potency and selectivity toward breast CSCs in vitro. Strikingly, **Pt-3** exhibits significantly greater breast CSC potency than salinomycin, cisplatin, and carboplatin in monolayer and three-dimensional cell cultures. The tri-nuclear complex, **Pt-3** induces breast CSC apoptosis by entering breast CSCs in relatively large quantities, bypassing the nuclear membrane, and inducing genomic DNA damage. As far as we are aware, this is first report of a platinum-based multi-nuclear complex with promising anti-CSC activity. Our findings reinforce the therapeutic potential of multi-nuclear agents and more specifically provides the basis for their development as breast CSC-selective agents.

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Keywords: platinum • cancer • DNA damage • multi-nuclear metal complexes• antitumor agents

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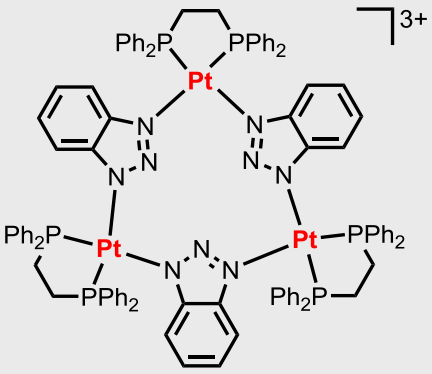

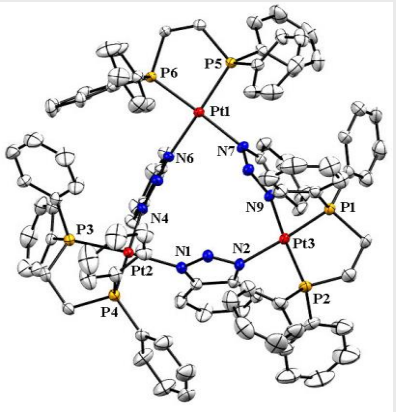

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Title

Killing cancer stem cells with platinum triangles: A multi-nuclear triangle complex with platinum(II) atoms at each corner is reported, and its breast cancer stem cell (CSC) potency and mechanism of cytotoxicity is described in detail. The triangle complex kills breast CSCs by inducing genomic DNA damage and triggering caspase-dependent apoptosis.

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